
Effect of ProRoot® MTA mixed with chlorhexidine on apoptosis and cell cycle of fibroblasts and macrophages *in vitro**

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Abstract

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Aim To compare the percentage of apoptotic cells and the cell cycle profile of fibroblasts and macrophages exposed to either ProRoot® mineral trioxide aggregate (MTA) mixed with chlorhexidine (CHX), or exposed to ProRoot® MTA mixed with sterile water.

Methodology Mouse gingival fibroblasts or mouse macrophages were seeded in six-well plates and allowed to attach overnight. Freshly mixed or set (allowed to dry for 24 h) specimens of tooth-coloured (white) ProRoot® MTA were prepared with 0.12% CHX gluconate (MTA/CHX) or with sterile water (MTA/H₂O). The cells were exposed for 24 h to the MTA specimens, which were placed over permeable membrane inserts to avoid direct contact with the cells. Untreated cells served as controls. Propidium iodide staining followed by flow cytometry was used to evaluate the effects of ProRoot® MTA on cell apoptosis

and cell cycle. Statistical analyses were performed by one-way ANOVA followed by *post-hoc* tests with the use of the SigmaStat 2.0 software, and significance was determined at $P \leq 0.05$.

Results MTA specimens containing CHX induced apoptosis of macrophages and fibroblasts ($P < 0.05$). In contrast, no change in the proportion of apoptotic cells was observed when sterile water was used to prepare the specimens ($P > 0.05$). Cell cycle analysis showed that exposure to MTA/CHX decreased the percentage of fibroblasts and macrophages in S phase (DNA synthesis) as compared with exposure to MTA/H₂O ($P < 0.05$).

Conclusion This *in vitro* study demonstrated that the substitution of CHX for sterile water in MTA increases its cytotoxicity. This suggests that the potentially beneficial antimicrobial effect of CHX may be accompanied by an increase in the cytotoxicity of the resulting MTA-based material.

Keywords: biocompatibility, cell death, cytotoxicity, DNA replication, endodontics, periodontal ligament.

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Introduction

Since its introduction to endodontics in 1993, mineral trioxide aggregate (MTA) has gained acceptance as the

material of choice for several clinical procedures. It has been shown to be an effective pulp capping material (Faraco & Holland 2001). MTA has been used to seal and repair root perforations (Pitt-Ford *et al.* 1995, Holland *et al.* 1999), and to create an apical barrier in teeth with open apices (Witherspoon & Ham 2001). Success as a root-end filling material has also been reported (Bates *et al.* 1996). MTA seems to be an adequate material for these procedures because of its sealing ability when compared with other materials such as amalgam, IRM or SuperEBA (Torabinejad *et al.* 1995a, Fischer *et al.* 1998). *In vitro* and *in vivo* studies

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have reported that MTA is biocompatible with the surrounding tissues (Koh *et al.* 1998, Torabinejad *et al.* 1998). It was shown to promote osteoblast activity (Koh *et al.* 1997) and was less cytotoxic than amalgam, IRM or SuperEBA (Kettering & Torabinejad 1995, Keiser *et al.* 2000). Furthermore, MTA has been found to possess antibacterial activity (Estrela *et al.* 2000).

The major components of ProRoot® MTA (Dentsply Tulsa Dental, Tulsa, OK, USA) are tricalcium silicate and dicalcium silicate. It also contains traces of other compounds including bismuth oxide that is added to enhance its radiopacity. It is dispensed with a liquid component, sterile water, which is used to prepare the mix. Recently, white ProRoot® MTA (tooth-coloured formula) has become available. This new formulation does not contain iron, which is found in the grey MTA and which occasionally caused staining of teeth. Holland *et al.* (2002) have investigated the biocompatibility of the white MTA in rat subcutaneous tissue and have found similar results with those reported for the grey MTA formulation (Holland *et al.* 1999).

Chlorhexidine (CHX) is a cationic antimicrobial agent (Hauman & Love 2003). It has been widely used as an antiseptic and is active against Gram-positive and Gram-negative bacteria, facultative anaerobes and aerobes, moulds, yeasts and viruses (Davies 2003). Its action is by adsorbing onto the cell wall of the microorganism and causing leakage of intracellular components and eventually leading to cell death (Leonardo *et al.* 1999). Oral CHX rinses, available in the United States in the form of 0.12% CHX gluconate (Peridex®, Zila Pharmaceuticals, Inc., Phoenix, AZ, USA; or Perigard®, Colgate Palmolive, New York, NY, USA), have been shown to be effective in decreasing plaque formation and controlling gingivitis (Yates *et al.* 2002). In endodontics, CHX has been found to be an effective antimicrobial agent when used as a root canal irrigant (Delany *et al.* 1982). However, *in vitro* and *in vivo* studies have demonstrated that CHX is potentially toxic to human cells and tissues (Bassetti & Kallenberger 1980, Pucher & Daniel 1992) and its cytotoxicity is dose and time-dependent (Babich *et al.* 1995).

In the clinic, ProRoot® MTA is used in close proximity to sites of inflammation and tissue injury. Fibroblasts, the predominant cell population of the periodontal connective tissue, are the cells responsible for the formation and maintenance of the periodontal ligament attachments as well as being involved in the repair, remodelling, and regeneration of the adjacent bone and cementum (Barthold *et al.* 2000). Macroph-

ages, on the other hand, play an important role in inflammatory processes (immune defence, T-cell activation and immune response), and are considered important orchestrators of wound healing throughout the body (Polverini 1997). Therefore, it is believed that the function of these two cell populations is critical for the successful response of the host to the treatment of root perforations or to induce apexification of necrotic immature teeth.

Flow cytometry is a unique method of allowing both cell cycle analysis and apoptosis detection. Apoptosis, or programmed cell death, occurs under normal physiological conditions and the cell is an active participant in its own demise (Kerr *et al.* 1972). Apoptosis also occurs in response to exposure to toxic dental materials. This phenomenon was observed when dental pulp cells were exposed to bonding agents (Mantellini *et al.* 2003). Hence, the analysis of apoptosis can be used as an additional surrogate marker for the cytotoxicity of dental materials.

A recent study reported an enhanced antimicrobial activity by the substitution of 0.12% CHX gluconate for sterile water in ProRoot® MTA (Stowe *et al.* 2004). This could be a potential improvement to the overall therapeutic effect of MTA in endodontics. However, the effect of adding CHX to the mixture of MTA on the survival and cell cycle of fibroblasts and macrophages remains to be determined. The purpose of this study was to compare the percentage of apoptotic cells and the cell cycle profile of fibroblasts and macrophages exposed to either ProRoot® MTA mixed with CHX or exposed to ProRoot® MTA mixed with sterile water.

Materials and methods

Cell culture

Mouse gingival fibroblasts (gift from C.T. Hanks, University of Michigan) and mouse macrophages (RAW 264.7; ATCC, Manassas, VA, USA) were cultured in Dulbecco's modified eagle's medium (DMEM; Invitrogen, Carlsbad, CA, USA) supplemented with 10% foetal bovine serum, 250 µg mL⁻¹ L-glutamine, 125 U mL⁻¹ Penicillin, and 125 µg mL⁻¹ Streptomycin (Gibco, Grand Island, NY, USA), at 37 °C in a humidified CO₂ incubator.

MTA specimens

White ProRoot® MTA (100 mg, tooth-coloured formula; Dentsply Tulsa Dental) were mixed with 35 µL

sterile distilled water (MTA/H₂O) according to the manufacturer's instructions. Alternatively, 100 mg ProRoot® MTA were mixed with 35 µL Peridex® (Zila Pharmaceuticals) (MTA/CHX). The size of each resultant pellet was approximately 5 mm × 5 mm. In one set of the specimens, the fresh pellet was immediately placed over a transwell insert measuring 24 mm in diameter (Corning, New York, NY, USA) that fits one well of a six-well cell culture plate (Corning), as depicted (Fig. 1). The transwell insert contains a permeable membrane (0.4 µm-pore size) and was used to prevent direct physical interaction between the cells and the specimens while allowing for soluble compounds from the specimens to reach the cells (Fig. 1). In another set of specimens, the pellet was allowed to set for 24 h at 37 °C in a humidified CO₂ incubator prior to placement onto the transwell insert, as described above. Untreated controls were cells cultured in a six-well plate, with transwell inserts, but without the MTA specimen.

Flow cytometry

Propidium iodide staining followed by flow cytometry was used to evaluate the effects of MTA/CHX and MTA/H₂O on cell viability and cell cycle, as described (Nör et al. 2002, Mantellini et al. 2003). Fibroblasts (1.5×10^5 cells/well) or macrophages (1.0×10^5 cells/well) were seeded in six-well plates (Corning) containing 2 mL of culture medium and allowed to attach overnight. The cell culture medium was replaced and the cells were then exposed to the MTA specimen placed over the membrane insert for additional 24 h, in triplicate wells per condition. Three wells per condition and cell type were evaluated in each experiment. At the end of the treatment period, both attached and floating cells were harvested, centrifuged and resuspended in a hypotonic solution containing 50 µg mL⁻¹ propidium iodide (Sigma, St Louis, MO, USA), 0.1% sodium citrate, 0.1% Triton X, and 100 µg mL⁻¹ RNase A. Samples were incubated in the dark for 30 min at 4 °C, and the proportion of

apoptotic cells was quantified by flow cytometry from 10^4 cells per well (EPICS; Beckman Coulter, Miami, FL, USA). Apoptotic cells were defined as cells in the Sub-G₁ phase of the cycle. Alternatively, cells were exposed to the same conditions as described above and cell cycle was evaluated with 'MPlus software' (Phoenix Plus Systems, San Diego, CA, USA). Three independent experiments were performed per cell type and treatment protocol.

Statistical analysis

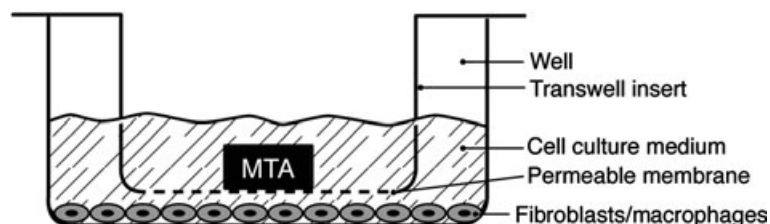
The statistical analyses of the data were performed by one-way ANOVA followed by a multiple comparison Tukey's test using SigmaStat 2.0 software (SPSS, Chicago, IL, USA). Statistical significance of the data was determined at $P \leq 0.05$.

Results

Fresh MTA prepared with CHX induced apoptosis of fibroblasts and macrophages

The degree of cytotoxicity of the fresh and the set mixes of MTA/CHX and MTA/H₂O on macrophages and fibroblasts was determined as a function of the percentage of apoptotic cells (Fig. 2). It was observed that the fresh MTA/CHX specimens induced an increase in the percentage of apoptotic macrophages and fibroblasts when compared with the MTA/H₂O group or the untreated controls ($P \leq 0.05$) (Fig. 2a,c). In contrast, the set MTA/CHX specimens did not show significant differences in the percentage of apoptotic fibroblasts or macrophages ($P > 0.05$) (Fig. 2b,d). Interestingly, we did not observe a significant difference in the percentage of apoptotic cells when the MTA/H₂O specimens were compared with the untreated controls ($P > 0.05$). These results indicate that the fresh mixture of MTA/CHX induced a modest increase in the percentage of apoptotic macrophages and fibroblasts. We did not observe noticeable micromorphological changes in fibroblasts or macrophages following a 24-h

Figure 1 Schematic drawing of the transwell insert used in the six-well plates to prevent the direct physical contact between the cells (macrophages or fibroblasts) and the MTA specimens.



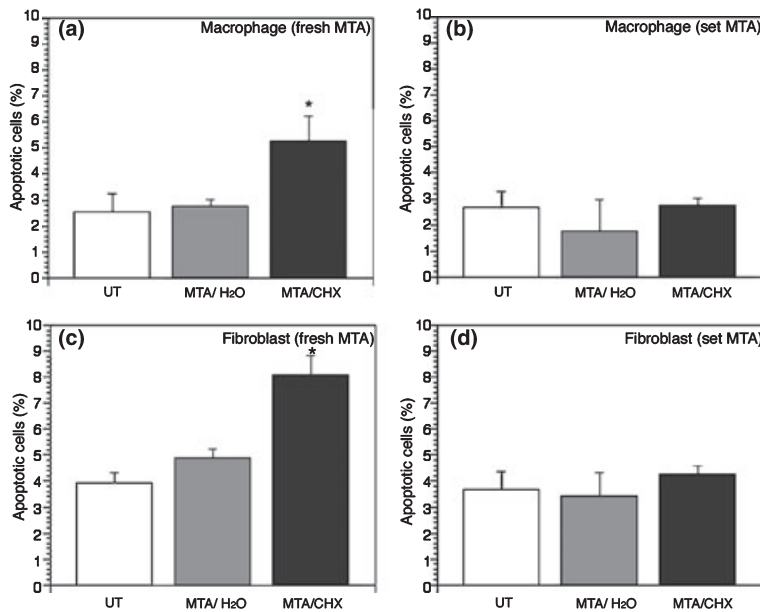


Figure 2 Fresh specimens prepared with ProRoot[®] MTA and chlorhexidine induced apoptosis of fibroblasts and macrophages. Percentage of apoptotic macrophages (a, b) and fibroblasts (c, d) after a 24-h exposure to fresh or set specimens of MTA. Solid grey bars depict MTA/H₂O and solid black bars depict MTA/CHX. Apoptotic cells were identified after staining with propidium iodide and sorting by flow cytometry. Asterisk indicates statistical significance at $P \leq 0.05$, as compared with untreated controls (white bars). Data represent mean values (\pm SD) of triplicate samples per condition and cell type.

exposure to MTA/CHX under light microscopy at 200 \times (Fig. 3).

MTA prepared with CHX induced a decrease in the proportion of fibroblasts and macrophages in the S phase of cell cycle

To assess whether MTA/CHX causes changes in the cell cycle and cell proliferation, flow cytometry was performed using the same conditions as mentioned above. It was observed that exposure of macrophages or fibroblasts to the fresh or set specimens of MTA/CHX mediated an increase in the proportion of cells in the G₁ phase of the cell cycle when compared with the untreated controls ($P \leq 0.05$) (Fig. 4). This finding was associated with a decrease in the proportion of cells in the S phase of the cell cycle ($P \leq 0.05$). There were no significant differences observed in the proportion of cells in each phase of the cell cycle (G₁, S or G₂ phase) when fresh or set MTA/H₂O specimens were compared with untreated controls ($P > 0.05$).

Discussion

The evaluation of the cytotoxic potential of materials used in root canal treatment is of clinical relevance because these materials may cause degeneration of the adjacent periapical tissues and delay wound healing. Ideally, a root-end filling material is preferably bactericidal, or at least bacteriostatic, and yet is well tolerated by the periapical tissues (Rubenstein 2001). The addition of the antimicrobial agent CHX to MTA has been evaluated in a recent report (Stowe *et al.* 2004). However, the effect of MTA/CHX on the viability of cells that are involved in the regeneration of periapical tissues is still not fully understood.

The cytotoxicity of CHX has been reported in *in vivo* and *in vitro* studies (Yesilsoy *et al.* 1995, Chang *et al.* 2001). Therefore, it was hypothesized that the substitution of 0.12% CHX gluconate for sterile water in MTA could induce apoptosis of fibroblasts and macrophages (cell populations that can potentially be affected during the clinical use of MTA in endodontic

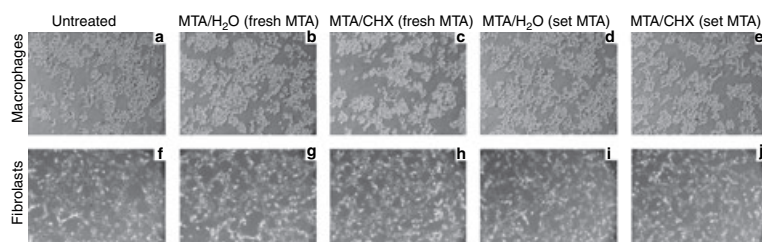


Figure 3 Micromorphology of macrophages and fibroblasts exposed to fresh MTA/H₂O (b, g); fresh MTA/CHX (c, h); set MTA/H₂O (d, i) and set MTA/CHX (e, j). Untreated controls (a, f) are shown. All photomicrographs were taken at 200 \times magnification.

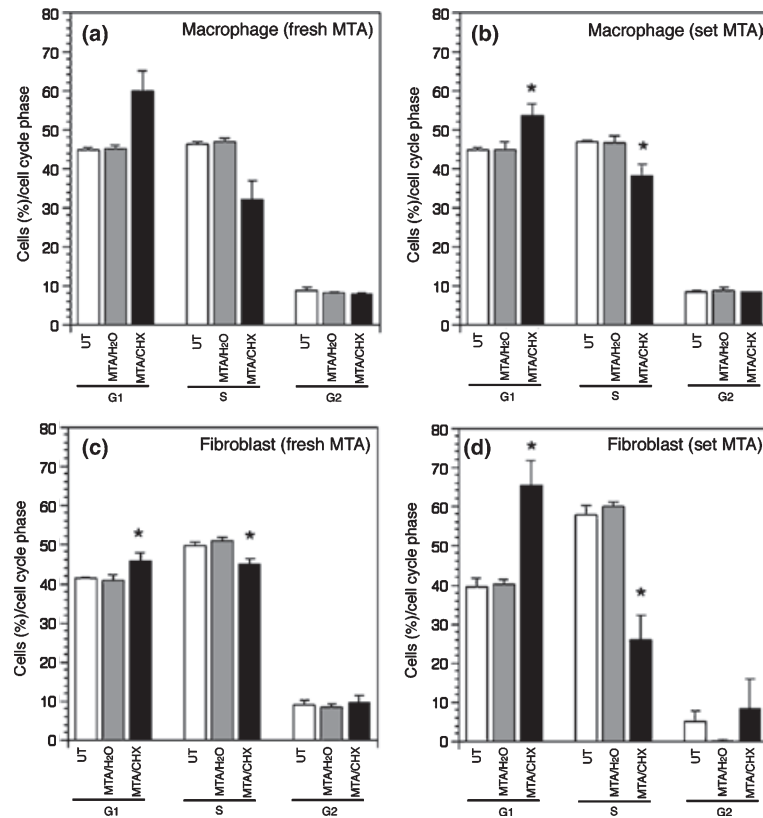


Figure 4 Specimens prepared with ProRoot® MTA and chlorhexidine induced a decrease in proportion of cells in active synthesis of DNA (S phase). Flow cytometry profile of macrophages (a, b) or fibroblasts (c, d) exposed to fresh and set specimens of MTA and stained with propidium iodide. Solid white bars depict untreated control cells, solid grey bars and solid black bars depict percentage of cells exposed to MTA/H₂O and MTA/CHX, respectively. Asterisk indicates statistical significance at $P \leq 0.05$, as compared with untreated controls (white bars). Data represent mean values (\pm SD) of triplicate samples per condition and cell type.

treatments). A modest (but significant) increase in the percentage of apoptotic cells when macrophages or fibroblasts were exposed for 24 h to freshly mixed MTA/CHX was observed, compared with exposure to MTA/H₂O specimens. As the MTA/H₂O specimens did not induce apoptosis above the levels observed with the untreated controls, this finding may be interpreted as an increase in cytotoxicity mediated by the solution containing 0.12% CHX gluconate used to prepare the MTA/CHX specimens.

Interestingly, a noticeable change was not observed in the morphology of the cells exposed to MTA/CHX upon microscopic examination. This is surprising as the hallmarks of apoptotic cell death are the blebbing and detachment of the cells, and budding of the whole cell to produce membrane-bounded round bodies (Kerr *et al.* 1972). A reasonable explanation for this finding is that the relative percentage of apoptotic cells in the cultures exposed to MTA/CHX was up to 8% after 24 h. While this represents approximately a twofold increase in the percentage of apoptotic cells as compared with controls, it may not be sufficiently high to be clearly distinguishable under light microscopy. In addition, it should be noted that despite the fact that MTA/CHX induced a

statistically significant increase in the percentage of apoptotic cells in this *in vitro* study, the cytotoxic effects of MTA/CHX *in vivo* remain to be determined, and will be the subject of future investigation.

In an attempt to understand the effect of CHX added to MTA on fibroblast and macrophage proliferation, a cell cycle analysis was performed. The cell cycle analysis showed that MTA/CHX induced an increase in the proportion of macrophages and fibroblasts in the G₁ phase with an associated decrease in the proportion of cells in the S phase. As the S phase of the cell cycle is the phase in which DNA is replicated and the cell prepares for mitotic division, these data demonstrate that CHX used in combination with ProRoot® MTA has an anti-proliferative effect for these two cell types *in vitro*. Therefore, our results suggest that MTA/CHX might affect the regeneration of the periapical tissue negatively following its use in endodontics, as this process is partially dependent upon local proliferation of fibroblasts and macrophages.

In the present *in vitro* investigation, the white ProRoot® MTA prepared with sterile water proved not to be cytotoxic to macrophages and fibroblasts in both fresh and set mixes. This is in general agreement with

previous cytotoxicity studies of the grey ProRoot® MTA by Torabinejad *et al.* (1995b)) and Osorio *et al.* (1998) and the white ProRoot® MTA by Holland *et al.* (2002). Therefore, the recent change in the formulation of this material that resulted in the introduction of the white ProRoot® MTA in the market does not seem to have affected its biocompatibility, as long as it is prepared with sterile distilled water.

Although the use of CHX in combination with MTA has clearly enhanced the antimicrobial properties of the material (Stowe *et al.* 2004), our findings suggest that the resulting mix (i.e. MTA/CHX) is more toxic to the host cells. It is speculated that MTA/CHX may have an unfavourable effect on the resolution of the periapical periodontitis, and the regeneration of the periodontal connective tissue attachment apparatus. Further investigations should be performed to evaluate if the cytotoxic effects of MTA/CHX observed here are also manifested when this mixture is utilized *in vivo*.

Conclusions

This *in vitro* study showed that the fresh mix of ProRoot® MTA with 0.12% CHX gluconate induced apoptosis of macrophages and fibroblasts. Moreover, the ProRoot® MTA mixed with sterile water did not induce apoptosis of these cells. Taken together, these findings suggest that the potentially beneficial antimicrobial effect of CHX may be accompanied by an increase in the cytotoxicity of the resulting MTA-based material.

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